ACETYLCHOLINE AND NERVOUS INHIBITION IN THE HEART OF VENUS MERCENARIA

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During a study of the comparative pharmacology of the hearts of invertebrate animals the students in the physiology course at the Marine Biological Laboratory [2] have found the heart of the quahog, *Venus mercenaria,* to be extremely sensitive to acetylcholine. Jullien showed (see Jullien, 1936, for summary and references) that the hearts of several mollusks are inhibited by acetylcholine. He found that atropine does not antagonize this inhibition.

The Clam Heart as a Test Material for Acetylcholine

The sensitivity of the heart of *Venus* to acetylcholine is such and the preparation is so stable that it has some advantages over the commonly-used leech muscle as a test material for acetylcholine. The heart can be tested *in situ;* or the heart can be mounted in a small tube of sea water which can be replaced by the test fluid; or the heart can be mounted in a chamber through which test fluid is perfused. When test solutions are dripped on the heart *in situ* the threshold is many times higher than by the other methods. We have found the perfusion chamber method most satisfactory and have used several types of chamber. The fluid enters through a tube at the bottom and an overflow tube at the top is at the level of the surface of the water in a constant temperature bath. In some experiments the heart is mounted either by ligatures around the ends of the ventricle or by tiny glass hooks and the fluid surrounding the heart slowly changed from aspirator bottle reservoirs. In other experiments the heart is perfused directly in the chamber through a cannula which enters along the intestine into the ventricle and holds the heart

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[2] Some of the experiments reported here were first done in the Physiology Course at the Marine Biological Laboratory. I am grateful to all those students who have been interested in this problem during four summers. I also wish to acknowledge the help of Hazel B. Prosser in verifying and extending the experiments.

at the lower end. The perfused hearts show a quicker response to test materials than those in circulating medium.

The beat of the heart is very sensitive to tension and to rate of flow. Frequently a heart fails to beat when first mounted, but when a slight tension is applied the beat starts regularly. It is very important to keep the tension and the rate of flow through and around the heart constant. Indeed Koehring (1937) has found that under natural conditions the heart of *Mya* does not beat when no fluid is flowing through the mantle cavity. In general, increasing the rate of flow increases the amplitude of the beat, decreasing the rate of flow diminishes the amplitude of beat. We normally count drops from the overflow of the chamber and keep the rate at approximately two drops per second.

The stability of the preparation depends partly upon the temperature. The sensitivity to acetylcholine declines slightly with time but this decline is much slower below 20° C. than above that temperature. We have frequently worked with preparations during a period of 24 hours or longer at 15–20° C. Normally a heart mounted in the morning retains high sensitivity throughout the day.

Sea water has ordinarily been used as a perfusion fluid, but in experiments done at an inland laboratory in the winter Van't Hoff's solution of artificial sea water has been found entirely satisfactory. Acetylcholine diluted in sea water retains its potency for four to six hours after which a decline in its activity can be observed.

The sensitivity of the hearts to acetylcholine and their stability as long-lasting preparations is much greater if the clams are freshly dug. Clams which have been kept either in running sea water or in a refrigerator for a week are much less satisfactory than those which have been dug within a day or two.

The sensitivity of the heart of *Venus* to acetylcholine shows seasonal variation. We have made tests at different times of the year during three years on over a hundred specimens. The sensitivity is highest in the spring. From March to early July marked inhibition occurs with dilutions as great as 10^{-12} to 10^{-11}. Occasional preparations show even greater sensitivity. Sensitivity can be increased slightly by eserinization. Late in the summer and during the fall and early winter the sensitivity is low and thresholds are of the order of 10^{-10} to 10^{-9}. This variation, both seasonal and among individual clams, is much greater than has been reported for the leech muscle which usually shows a threshold after eserinization of 10^{-9}.

The beat of the *Venus* heart usually ceases completely at a concentration ten times as great as that which slightly decreases the amplitude. The leech muscle gives differences in contraction over a concentration

range of several logarithmic units, hence it is more useful for testing different concentrations of acetylcholine. The advantages of the *Venus* heart are its ease of preparation, its constancy of response over periods of many hours, its high sensitivity and resulting ability to detect traces of acetylcholine. The *Venus* heart provides material useful for acetylcholine bio-assay if attention is paid to the factors of tension, rate of perfusion, temperature, freshness of the preparation, age of the acetylcholine dilution, and season of the year.

The Effects of Drugs

Acetylcholine has primarily a negative inotropic action on the clam heart (Fig. 1) although frequently a slight slowing also occurs (Fig.

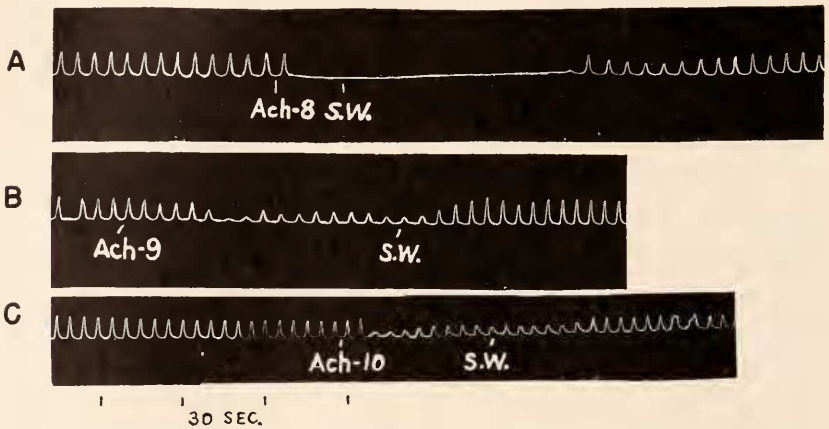

FIG. 1. *A*, effect upon a *Venus* heart in a chamber of acetylcholine 10^{-8} followed by sea water (S.W.). *B* and *C*, effects of acetylcholine 10^{-9} and 10^{-10} respectively. Late July preparation.

5, *A*, *B*). The threshold for the reduction in amplitude is slightly lower than that for the reduction in rate. When perfusion continues with a threshold concentration of acetylcholine, some recovery from the initial depression may occur. When higher concentrations are used the beat is stopped in diastole, a slight fall in tonus may occur, and no recovery occurs until the acetylcholine is removed.

Previous treatment with eserine (10^{-4}) for a period of 15 to 30 minutes increases the magnitude of the acetylcholine effect (Fig. 5*B*). The sensitivity to acetylcholine may be increased several times but the most marked effect is the prolongation of the inhibition.

Jullien found that atropine makes the beat of snail and oyster hearts irregular. We have found atropine to be extremely toxic to the heart

of *Venus*. In concentrations of 10^{-4} or 10^{-5} it not only makes the beat irregular but frequently stops the heart (Fig. 2, *A*, *B*). Preparations differ widely in their sensitivity to atropine. When non-toxic concentrations of atropine are used in combination with or preceding treatment with acetylcholine there is no consistent antagonism of the acetylcholine

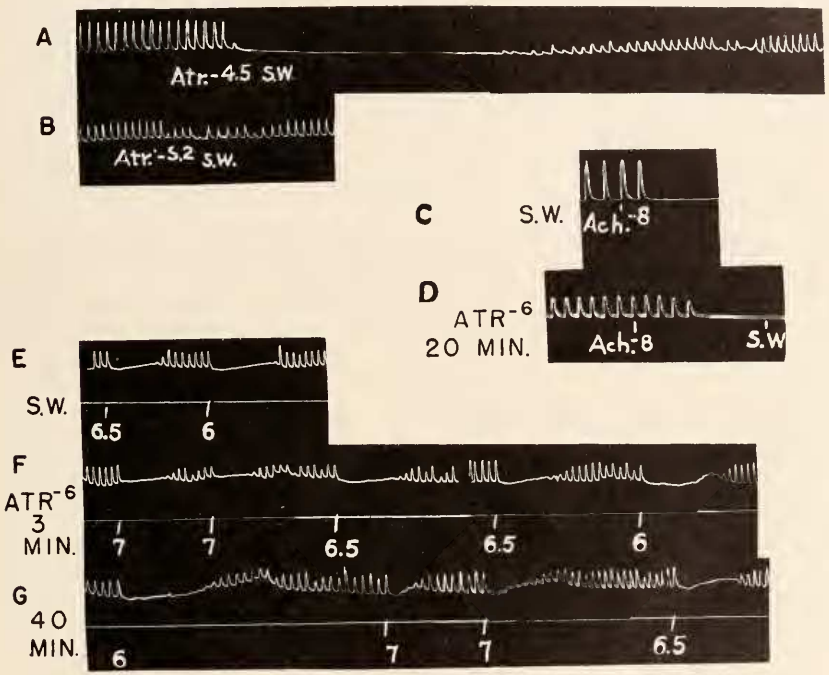

FIG. 2. Three experiments on the effect of atropine on the heart of *Venus*. *A*, toxic effect of atropine sulphate $10^{-4.5}$ followed by sea water. *B*, toxic effect of atropine sulphate $10^{-5.2}$ followed by sea water. *C*, another preparation, effect of acetylcholine 10^{-8} before and *D*, after bathing with a non-toxic concentration of atropine, showing lack of antagonism. The slight delay in inhibition after atropine is within the normal variation of sensitivity to acetylcholine. *E*, *F*, *G*, inhibition by visceral ganglion stimulation in another preparation. Numbers give position of secondary of inductorium. *E*, in sea water, *F*, after treatment with atropine sulphate 10^{-6} for 3 minutes and *G* for 40 minutes. Atropine 10^{-5} was found very toxic for this preparation.

inhibition (Fig. 2, *C*, *D*). This must mean either that any possible antagonism is masked by the toxic effects or else that the receptor mechanism for acetylcholine inhibition is different from that in the vertebrate heart and other systems where atropine antagonism is found.

Adrenaline increases the frequency of the heart beat and to a slight extent the tonus of the heart. In high concentrations it may cause cessation of beat in systole.

Nicotine acts much like acetylcholine in causing a reduction in amplitude and arrest in diastole.

Manner of Action of Acetylcholine on the Heart of Venus

Acetylcholine acts in opposite manner upon the hearts of mollusks and arthropods. In the mollusks its effect is always inhibitory as in the vertebrates. In the arthropods, however, it is an accelerator (cf. Welsh, 1939*a, b* on several decapods; Hamilton, 1939, on the grasshopper). The threshold for the accelerating action on arthropod hearts is higher than that for inhibition in the molluscan hearts.

Histological examination shows nerve cells in the hearts of all arthropods examined but in only a few mollusks (cf. Alexandrowicz, 1912). Miss Audrey Smith in the 1938 Physiology Class at the Marine Biological Laboratory has made a careful histological study of the hearts of the crab *Libinia* and the clam *Venus* by methylene blue, toluidin blue, and silver techniques. Her preparations show nerve cells in the spider crab heart and in the ganglia of the clam; they show nerve endings but no nerve cells in the heart of *Venus*.

When examined microscopically a weakly beating heart of *Venus* shows the contraction originating for successive beats at different points on the heart. It is difficult to see this in a strongly beating heart. These lines of evidence indicate, therefore, that the contraction of the heart of *Venus* is strictly myogenic in contrast to arthropod hearts and suggest that acetylcholine acts directly on heart muscle in this animal. In the heart muscle itself there must occur pacemaker, conduction and contraction processes.

We have examined microscopically the living hearts of clams before and after the beat is stopped by acetylcholine. When completely inhibited, a heart can readily be excited to single contractions by local mechanical or electrical stimulation. These contractions are sometimes localized and sometimes spread through the entire heart. This must mean that the contractile mechanism and sometimes the conductile mechanism also are intact. No rhythmic action potentials are present in an inhibited heart. During recovery from acetylcholine inhibition little beats may occur locally at scattered points over the heart, but they do not spread to surrounding regions. It is as if the pacemaker stimulus is either too weak to set up a general wave of excitation or else the wave is not conducted normally. The fact that the principal action is a negative inotropic one indicates an upset in conduction although the slight chronotropic action suggests an effect upon the pacemaker mechanism also. It seems likely, therefore, that acetylcholine acts either upon the

pacemaker processes or the conduction mechanism or both rather than upon the mechanism of contraction.

Mechanism of Normal Cardiac Inhibition

It has been demonstrated by Carlson (1905) and Budington (1904) that stimulation of the visceral ganglion of the clam causes inhibition of the heart. The effect of stimulating at several different intensities is shown in Fig. 3*A*. The gills on the left side were cut and rigidly mounted electrodes were placed in contact with the visceral ganglion which lies on the inner side of the posterior adductor muscle. Thus the electrodes remained in constant position for many hours. Motley (1934) maintained that such depression of beat as he obtained with freshwater mussels on ganglionic stimulation might be due to movements of the foot and visceral mass. This is most certainly not true in our experiments since the inhibition occurs clearly when the heart is lifted up away from the visceral mass except at its two ends.

As Budington pointed out, the duration of the inhibition far outlasts the nerve stimulation and there may be cardiac escape if a weak stimulation continues. The threshold of this inhibition decreases slightly during the first half-hour after a clam is prepared. It then remains constant for many hours. Similar arrest of the heart occurs reflexly if the mantle or foot is pinched (Fig. 3*C*). If a series of tetanic stimuli are applied to the visceral ganglion at 20-second intervals the later bursts in the stimulation series are less effective than earlier ones at the same intensity (Fig. 3*B*). If, however, one or two minutes elapse between stimulation periods, the response remains very constant. If the inhibition be due to a liberated mediator this effect might be due to the requirement of time for resynthesis of the mediator in the nerve axon terminations.

The effect of acetylcholine upon the heart can very easily be duplicated in duration and magnitude by stimulating the visceral ganglion. It is possible, therefore, that acetylcholine acts as an inhibitory mediator here as it does in the vertebrates.

Jullien (1936) argued that because atropine does not antagonize the acetylcholine inhibition of the mollusk heart, a cholinergic inhibitory mechanism is probably not present. It seems to us unjustified to argue from drug action to nervous mechanism. Two tests must be applied to any theory of chemical mediation: the presence of the active substance should be demonstrated following nerve stimulation, and the effects of drugs should be combined with nerve stimulation.

Transfer of fluid from an intact clam heart subjected to nerve inhibition by visceral ganglion stimulation to another heart either in a small chamber or perfused *in situ* has been attempted in several experiments on each of eleven preparations. Direct perfusion of the inhibited heart

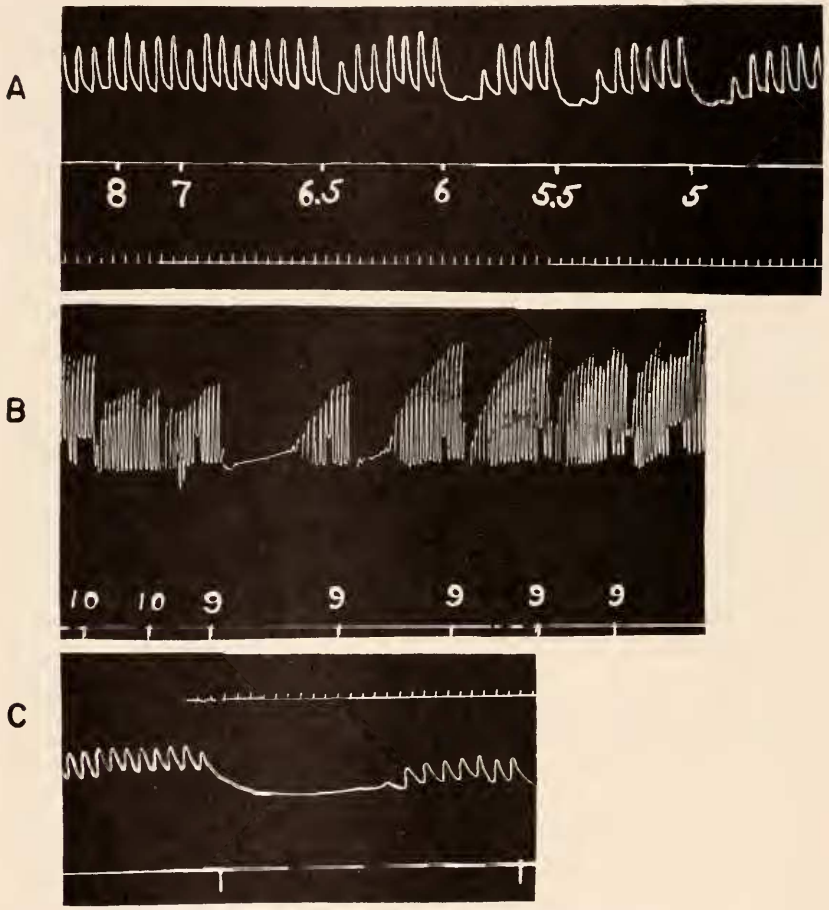

FIG. 3. *A*, effect of brief tetanic stimulation of visceral ganglion at intensities indicated by position in centimeters of inductorium secondary coil. *B*, effect of repeated frequent stimulation at same intensity (9 cm.). *C*, reflex inhibition; the mantle was pinched at time indicated by the signal. Time intervals in *A* and *C*, three seconds.

is very difficult and it is best to fill the pericardium in which the punctured heart lies with sea water containing eserine. Intermittent stimulation of the visceral ganglion is then carried out for thirty seconds during which the heart is at a standstill. The two cubic centimeters of

fluid bathing the heart are then transferred to the test heart. In some experiments there was no effect. Eight of the eleven preparations, however, showed some reduction in amplitude of beat of the test heart when bathed by fluid from an inhibited heart (Fig. 4*C*). When eserine was added to the sea water in the pericardium to prevent hydrolysis of any acetylcholine liberated and to the test heart for sensitization, the results were much more convincing than when no eserine was used. The amount of fluid that can be obtained from the pericardium and particularly the amount that can come in contact with the interior of the heart is very small. The results do, however, suggest that a substance is

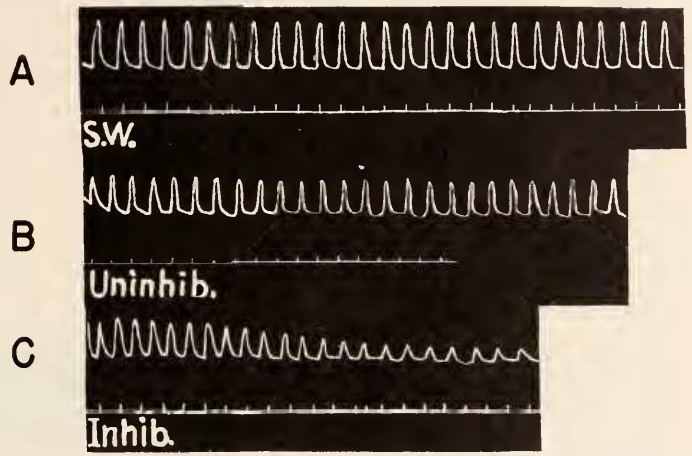

FIG. 4. The effect of fluid transferred from an *in situ* heart to a test heart in a small chamber. *A*, beat of test heart in sea water. *B*, transfer of eserinized sea water from pericardial cavity of heart beating normally. *C*, transfer of eserinized sea water from pericardium of same heart inhibited for 30 seconds by intermittent tetanic stimulation of the visceral ganglion. Time signal, 6 seconds.

liberated in the normally inhibited heart, is protected by eserine, and acts upon a test heart much like acetylcholine.

In a series of experiments drugs were combined with the nerve inhibition. Atropine in a non-toxic concentration was allowed to drip over a heart which was being inhibited by visceral ganglion stimulation. No decrease in the inhibition was obtained during many minutes of treatment with atropine (Fig. 2, *E, F, G*). This agrees with the fact that atropine does not antagonize the acetylcholine inhibition (Fig. 2, *C, D*).

The effect of eserine upon the nerve inhibition is shown in Fig. 5. When the heart is bathed with eserine (10^{-4} to $10^{-3.5}$) for 15 to 30 minutes it shows not only greater acetylcholine inhibition (Fig. 5*B*) but also more nerve inhibition (Fig 5*D*). The threshold for the nerve inhi-

bition is altered little if any but the duration of threshold inhibition is very greatly lengthened. In one experiment, for example, an inhibitory stimulus which before eserinization caused inhibition lasting approximately 30 seconds, after eserinization resulted in inhibition which lasted 50 minutes. Recovery from the effect of eserine is very slow; in these experiments it was appreciable after one-half hour but was not usually complete after several hours. These results indicate that the effect of

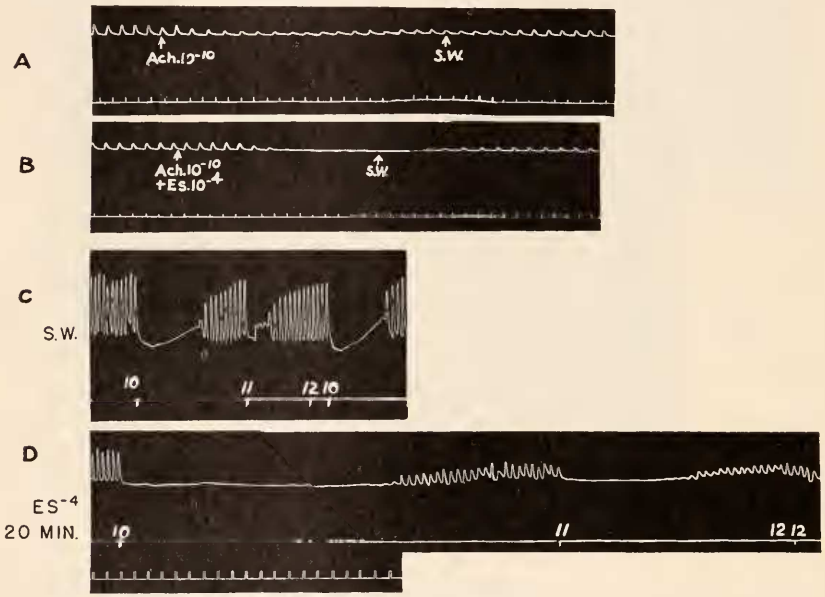

FIG. 5. Effect of eserine sulphate upon inhibition of the heart. *A* and *B* chamber preparation. *A*, inhibition by threshold concentration of acetylcholine 10^{-10}. *B*, inhibition due to same concentration of acetylcholine combined with eserine (10^{-4}) after eserinization for approximately 15 minutes. Eserine alone had no perceptible effect upon beat. *C* and *D* *in situ* preparation. *C*, inhibition of heart bathed with sea water by visceral ganglion stimulation at indicated positions of inductorium secondary. *D*, inhibition at same intensities of ganglion stimulation after 20 minutes of eserinization. Time interval in *A* and *B*, 3 seconds; in *C* and *D*, 10 seconds.

eserine is not to sensitize the heart but to prevent destruction of the inhibitory mediator. If acetylcholine is the mediator involved its action should be greatly prolonged because its hydrolysis by choline esterase is retarded. This is found to be true.

It is of interest that Bacq (1935) found a moderate amount of choline esterase in the blood of the pelecypod, *Pectunculus,* and in other molluscan tissues. He found several molluscan body muscles to respond

to acetylcholine, but Bacq and Coppée (1937) failed to get potentiation by eserine of responses of the foot of *Buccinium* or of *Mya.* Jullien, Vincent, Brouchet, and Viulet (1938) found very low acetylcholine and esterase contents in lamellibranch as contrasted with pulmonate mollusks. The very high sensitivity to acetylcholine, and the marked prolongation of inhibition by eserine agree with the low cholinesterase content of the heart of *Venus* as reported by Smith and Glick (1939). The slow recovery from the eserine effect indicates that the reactivation of the small amount of cholinesterase present must be a very slow process.

In summary, acetylcholine and nerve inhibition give similar pictures in *Venus;* in some experiments fluid transferred from inhibited eserinized hearts arrests test hearts; and eserine greatly prolongs both the inhibition due to acetylcholine and that due to visceral ganglion stimulation. These facts indicate that the normal inhibition in the heart of *Venus* may be by way of acetylcholine liberated at the terminations of nerve fibers from the visceral ganglion.

Summary

The heart of *Venus* is sensitive to acetylcholine in dilutions of 10^{-12} during the spring and 10^{-9} during the fall. It is useful as a test material for acetylcholine assay.

Acetylcholine appears to leave the contracting mechanism intact and to act on the pacemaker and conducting mechanisms of this myogenic heart.

Stimulation of the visceral ganglion causes inhibition in diastole resembling the effect of acetylcholine.

Atropine is very toxic to the heart. In non-toxic concentrations it antagonizes neither the effect of acetylcholine nor of nerve inhibition.

Fluid from a heart inhibited by visceral ganglion stimulation often depresses the beat of an eserinized test heart.

Eserine prolongs the inhibition due to acetylcholine and that due to nerve stimulation. It appears likely that acetylcholine is liberated as the normal cardiac inhibitory agent in *Venus.*

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